CROATICA CHEMICA ACTA
CCACAA **81** (3) 437–441 (2008)

ISSN-0011-1643
CCA-3262
Original Scientific Paper

Improved Performances of Polyacrylamide Gel by Addition of a New Type of Polymer: Detection of Allelic Losses

Marina Korolija,[a] Mirko Hadžija,[a] Pajica Pavković,[b] Sanja Kapitanović,[a] and Marijana Popović Hadžija[a,*]

[a]*Ruđer Bošković Institute, Division of Molecular Medicine, Bijenička c. 54, HR-10000 Zagreb, Croatia*

[b]*Vuk Vrhovac University Clinic, Dugi Dol 4a, HR-10000 Zagreb, Croatia*

RECEIVED DECEMBER 4, 2006; REVISED JUNE 4, 2007; ACCEPTED JUNE 6, 2007

The aim of this study was to improve detection of heterozygous samples and loss of heterozygosity using a new type of gel polymer. A total of 60 samples of normal and tumor colon tissue were tested. Elctrophoresis of amplified loci was performed on a standard acrylamide / *N,N*-methylenebisacrylamide gel, and the same gel containing Spreadex polymer, NAB (native acrylamide-bis). Standard gel displayed 36 samples of normal tissue as homozygous. Electrophoresis of the same samples on gel with addition of polymer revealed 12/36 heterozygous samples. Five of 12 matched tumor samples displayed loss of heterozygosity that could not be detected on standard gel, because of its lower resolving power. Electrophoresis on a new polymer presents a simple, inexpensive, reproducible and non-radioactive method for loss of heterozygosity detection, with improved performances such as increased sensitivity, decreased amount of a sample and improved separation of closely spaced bands. Application of the polymer may contribute to a vide variety of diagnostic procedures.

Keywords
DPC4
loss of heterozygosity
allele
Spreadex polymer NAB

INTRODUCTION

Electrophoresis on a standard polyacrylamide gel (PAGE) that contains monomers acrylamide / *N,N*-methylenebisacrylamide is a well established and commonly used method for detection of heterozygous samples and loss of heterozygosity (LOH) by microsatellite analysis (repeated DNA sequences around the gene).[1,2,3]

Loss of heterozygosity can indicate inactivating mechanism of tumor-suppressor genes that implies deletions of the relatively large chromosomal regions and possible gene loss. This is judged by comparing the intensity of electrophoretically separated DNA bands (representing alleles in cancer, and corresponding normal tissue of the same patient). LOH analysis allows the insight into the progress of carcinogenesis (from series of premalignant precursor lesions into invasive and metastatic cancer), and the possibility of detecting the disease at an early stage.[4,5,6] This method is therefore particularly useful tool in the cancer research.

Polyacrylamide gel has encountered technical difficulties regarding electrophoretic running time and resolution, as well as autoradiography detection, thus affecting interpretation of the results.[7,8,9,10] A need for an ac-

* Author to whom correspondence should be addressed. (E-mail: mhadzija@irb.hr)

curate, non-radioactive and quick method for LOH analysis has therefore emerged. It resulted with synthesis of new matrices for gel electrophoresis.

In this work we tested the potential of one new matrix (Spreadex Polymer, Native Acrylamide-Bis) in order to improve the power of acrylamide based gels to separate microsatellite fragments of slightly different length. We compared detection of heterozygous samples and the results of LOH analysis at two polymorphic regions flanking the *DPC4* gene obtained on the standard polyacrylamide gel, and the same gel containing Spreadex polymer NAB. Here we document the improvement of electrophoretic DNA separation efficiency that would influence not only the LOH analysis, but also a variety of research and diagnostic procedures.

EXPERIMENTAL

Cancer Specimens and DNA Extraction

For *DPC4* LOH analysis 60 surgical resection specimens from unselected colon adenocarcinoma patients were collected from Clinical Hospital Sestre Milosrdnice, Zagreb, Croatia. The diagnoses were confirmed histopathologically by pathologist. There were 31 men and 29 women, and the average age of the patients was 60.9 years, ranging from 30 to 75 years.

Haemotoxylin-eosin staining was performed to allow the assessment of tumor cell proportion that should be over 80 %. Normal colon mucous, more than 5 cm distant from the tumor, was taken as a source of corresponding normal DNA.

Fresh tissue was snap frozen in liquid nitrogen immediately after surgical removal and stored at –80 °C in a human tumor bank.[11] High molecular weight DNA was isolated from matched sample of normal and tumor tissue according to standard phenol-chloroform protocol.[12] Integrity of DNA was assessed on 1 % agarose gels with ethidium-bromide (Sigma, St. Louis, MO, USA), and DNA was quantified spectrophotometrically (260/280 nm) (M330W spectrophotometer; Camspec Ltd., Cambridge, UK).

Polymerase Chain Reaction

Amplification in polymerase chain reaction (PCR) was performed in a 25 μl mixture of 1 × HotMaster Taq Buffer (Tris-HCL, c = 20 mmol dm^{-3}, pH = 8.4; KCl, c = 50 mmol dm^{-3}; Eppendorf, Germany) with Mg^{2+} (c = 1.5 mmol dm^{-3}; Eppendorf, Germany) containing 100 ng of template DNA, 50 μmol dm^{-3} of each dNTP (Eppendorf, Germany) 5 pmol dm^{-3} of forward and reverse primers and 1 U HotMaster Taq DNA Polymerase (Eppendorf, Germany). (One unit is defined as the amount of enzyme that incorporates 10 nmol of deoxyribonucleotides (dNTPs) into acid insoluble form in 30 minutes at 74 °C.) Both non-tumor and tumor DNA was amplified using specific primers for two microsatellite loci (D18S46 and D18S474) which lay approximately 0.3 Mb from both sides of the *DPC4* gene.

Primers were as follows:

5'-GAATAGCAGGACCTATCAAAGAGC-3',

5'-CAGATTAAGTGAAAACAGCATATGTG-3' (D18S46) and

5'-TGGGGTGTTTACCAGCATC-3',

5'-TGGCTTTCAATGTCAGAAGG-3' (D18S474).

The PCR reaction was carried out in a Mastercycler personal (Eppendorf, Germany) at the following PCR conditions: 1 cycle of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 57 °C or 63 °C (depending on the primers), 30 s at 70 °C and the final extension 5 min at 70 °C. PCR products were loaded onto 1 % agarose gel with ethidium-bromide (Sigma, St. Louis, MO, USA).

Electrophoretic Separation

Normal and tumor specimens were analyzed for heterozygous profile and tested for loss of heterozygosity on two types of gels:

(i) Acrylamide / *N,N*-methylenebisacrylamide, 29:1 (Sigma) gel

PCR product (5–6 μl) of each sample was mixed with 1 μl of loading buffer (Eppendorf, Germany) and applied onto 12 % non-denaturig polyacrylamide gel, as it has been done before for analysis of DPC4 microsatellites.[13,14] Electrophoresis was performed in Hoefer EPS 3501 vertical system (Amersham Pharmacia Biotech, UK) at ambient temperature and under conditions given in Table I. The gels were silver-stained.

TABLE I. Comparison of conditions for electrophoresis performed on two types of gels

	12 % non-denaturing polyacrylamide gel	10 % non-denaturing polyacrylamide gel + Spreadex polymer NAB
Gel dimensions / cm	30 × 35 × 0.001	18 × 16 × 0.001
Optimal separation length / cm	17	8
PCR product per well / μl	6	1–2
Electrophoresis running time / h	18	3
Voltage / V	300	300
Buffer / mmol dm^{-3}	90 (TBE)	60 (TAE)

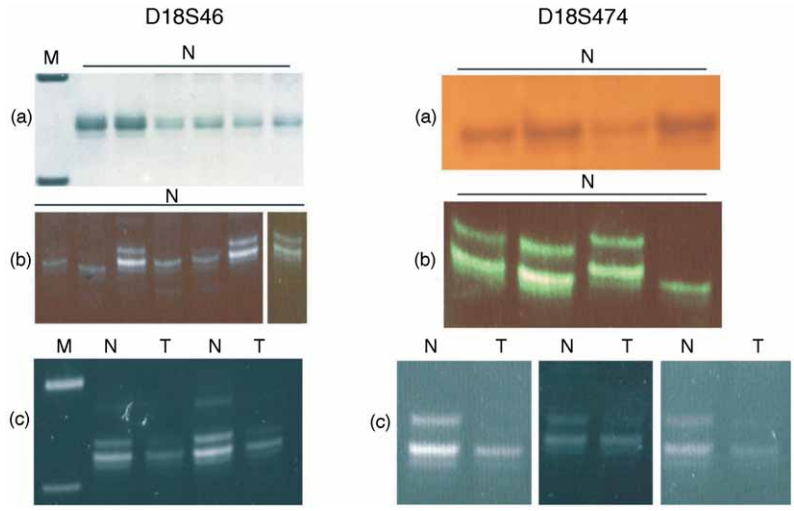

Figure 1. Loss of heterozygosity analysis at the DPC4 microsatellite loci D18S46 and D18S474. PCR products of normal colon tissues separated on 12 % non-denaturing polyacrylamide based gel (a) and 10 % polyacrylamide gel with Spreadex polymer NAB (b). Colon tumor samples with DPC4 loss of heterozygosity detected on 10 % gel with the polymer (c). Symbols: N, normal colon tissue; T, colon carcinoma tissue; M, molecular weight DNA marker pBR322 *Msp*I digest (BioLabs, New England).

(ii) Acrylamide / *N,N*-methylenebisacrylamide, 29:1 (Sigma) gel with addition of Spreadex polymer NAB

Samples that displayed homozygous pattern (one band) on polyacrylamide gels were additionally analyzed on 10 % non-denaturing polyacrylamide gels with 10 % (volume fraction) Spreadex polymer NAB (Elchrom Scientific, Switzerland). The gel was prepared in the same way as any standard polyacrylamide gel, and is consisted of two parts: upper 4 % gel containing sample wells but not NAB, and lower 10 % gel with addition of NAB, required for separation of DNA fragments.

Electrophoresis was carried out in Hoefer SE 400 Sturdier Vertical Units (Amersham Pharmacia Biotech, UK) at ambient temperature and under conditions given in Table I.

After electrophoresis, gels were stained with 1:10000 dilution stock 10000x SYBR Green I nucleic acid stain (Molecular Probes, Inc., USA) for 30 minutes, and analyzed by an UV transilluminator at 254 nm. Photographs were taken by direct screen camera (Polaroid DS-34).

LOH Assessment

LOH is defined as a complete absence or reduced signal by at least 50 % of one of the constitutional alleles in the tumor tissue, compared to the band of the corresponding non-neoplastic tissue. LOH was judged by visual examination of three investigators (M.P.H., S.K., and M.K.) and discordant results were re-evaluated after repeating the analysis.

RESULTS

DNA from 60 adenocarcinoma and normal mucous tissue samples was isolated for LOH analysis of *DPC4*. In order to validate standard PAGE genotyping, DNA of each pair was amplified with the primers for two markers (D18S46 and D18S474) that have been proven suitable for LOH analysis of the *DPC4* gene.[15,16,17,18]

A microsatellite analysis on 12 % standard polyacrylamide gels displayed 16 homozygous samples at the D18S46 locus (Figure 1a). However, the same samples analyzed on 10 % polyacrylamide gels with the Spreadex polymer NAB showed that three samples had two different alleles separated well (Figure 1b).

The amplification of the same region derived from corresponding tumor tissue was performed, and two matched PCR products were run in parallel lanes. LOH was observed in two tested samples (Figure 1c).

Second polymorphic site (D18S474) was amplified by corresponding set of primers on DNA template derived from normal tissue, and DNA fragments were submitted to electrophoresis on 12 % non-denaturing polyacrylamide gels. There were 20 samples showing merely one *DPC4* allele (Figure 1a). Electrophoresis of the same samples performed on a gel with the Spreadex polymer NAB showed that 9 samples exhibited two, closely but obviously separated alleles (heterozygous profile) (Figure 1b). In addition, in 3 matched tumor samples loss of one *DPC4* allele was observed (Figure 1c).

Results of LOH analysis performed on two types of gels at both microsatellite loci were summarized in Table II.

DISCUSSION

The analysis of polymorphic markers in acrylamide gels should demonstrate merely one (homozygous) or two (heterozygous) major bends, corresponding to alleles of analyzed gene. However, it has been shown that micro-

TABLE II. Results of DPC4-LOH analysis performed on 10 % non-denaturing polyacrlylamide gel + Spreadex polymer NAB of 36 samples that were read out as homozygous on 12 % standard polyacrylamide gel (16 samples at D18S46 and 20 samples at D18S474)

Sample	D18S46	D18S474
	N (*x* / %) [(a)]	*N* (*x* / %) [(a)]
Ho [(b)]	13 (81)	11 (55)
He [(b)]	3 (19)	9 (45)
LOH / He	2 / 3	3 / 9

[(a)] *N*, number of samples; *x*, amount fraction expressed in percents.

[(b)] Ho, Homozygous samples; He, Heterozygous samples.

satellites allelotypes of heterozygous frequently display more than two major bands, what can be due to different migration pattern of different conformational forms of the same DNA.[19] On the other hand, some heterozygous allelotypes can exhibit merely one band, if polymorphic sequences of two alleles differ in length slightly.

In order to overcome these problems, enhanced matrices for DNA electrophoresis, that improve specificity, sensitivity and resolution power of the gel, have been developed recently.

At the beginning of our study, precast Spreadex EL 300 and Spreadex EL 400 gels, proven effective in our previous DPC4-RFLP analysis,[14,20] were used to analyze dinucleotide repeats (D18S474 and D18S46) flanking the *DPC4* gene. As the profiles of representative samples in these gels were not satisfactory, the manufacturer was requested to repeat the analysis. The bends were diffused in both cases (data not shown). Although high resolution of dinucleotide microsatellite markers (chromosome 7q31.1) on Spreadex EL 300 gels has been demonstrated,[7] it could be that these types of gels are inappropriate for our particular *DPC4* microsatellite analysis.

In this work we used one new generation matrix (Spreadex polymer NAB) in order to increase the electrophoretic separation power of acrylamide based gel.[21] We continued our experiment analyzing results on standard 12 % acrylamide based gel. However, it became clear that the microsatellite dinucleotide repeats (D18S46 and D18S474) could be more clearly and accurately determined on 10 % PAA gel containing Spreadex polymer NAB. Namely, we proved that 19 % of samples at the D18S46 locus and 45 % of samples at the D18S474 locus remained undetected as heterozygous on 12 % polyacrylamide gel (falsely genotyped as homozygous), while two sharp, closely spaced, but fully separated bands distinctly emerged on 10 % gel containing the polymer. Moreover, besides detection of heterozygosity in normal tissue, improved resolution enabled also detection of loss of heterozygosity in colon adenocarcinoma. This feature can easily remain unspotted if the separation of closely spaced bands in gel fails. One group of authors also described good characteristics of gel with addition of NAB in analysis of short insertions (5382insC) and deletions (185delAG) in coding region of *BRCA* 1 gene, in samples of breast and ovarian tissues.[22]

In addition, polyacrylamide gel with the polymer requires shorter time for gel preparation, shorter electrophoresis running time (that corresponds with lower amount fraction of acrylamide-bisacrylamide) and lower amount of amplified product needed for relevant genetic analysis. These properties make this type of gel useful even when fragments (differing by ten or more base pairs) should be separated.

Here we report the advantages in application of a novel gel material (Spreadex polymer NAB) for gel electrophoresis. Compared to standard polyacrylamide gel, addition of the polymer to polyacrylamide solution enhanced gel resolution and decreased electrophoresis running time. Namely, we identified more samples of colon tissue that were tested for two different microsatellite repeats of the *DPC4* gene as heterozygous. Erill and coworkers[23] also described the problems in the evaluation of results following non-denaturing polyacrylamide gel electrophoresis of the same polymorphic markers in colon cancer tissues.

Thus, we can conclude that application of polyacrylamide gel with the Spreadex polymer NAB represents inexpensive system that can improve not only detection of closely spaced bends in polymorphism and mutational studies, but also a variety of research and diagnostic methods which require separation of DNA fragments that are slightly different in length.

Acknowledgements. – This work was supported by project grants 098-0982464-2460 and 098-0982464-2508 from the Ministry of Science, Education and Sports of the Republic of Croatia. We thank Mr. Sc. Mirjana Kozulić and Dr. Branko Kozulić for Spreadex polymer NAB. We also thank Marina Marš and Iva Pešun for skilful technical assistance.

REFERENCES

1. T. Toda, H. Oku, N. M. Khaskhely, H. Moromizato, I. Ono, and T. Murata, *Cancer Genet. Cytogen.* **126** (2001) 120–127.
2. S. Kapitanović, T. Čačev, M. Berković, M. Popović Hadžija, S. Radošević, S. Seiwerth, Š. Spaventi, K. Pavelić, and R. Spaventi, *J. Clin. Pathol.* **57** (2004) 1312–1318.
3. K. Pavelić, Š. Križanac, T. Čačev, M. Popović Hadžija, S. Radošević, I. Crnić, S. Levanat, and S. Kapitanović. *Mol. Med.* **7** (2001) 442–453.
4. E. R. Fearon and B. Vogelstein, *Cell* **61** (1990) 759–767.
5. K. W. Kinzler and B. Vogelstein, *Cell* **87** (1996) 159–170.
6. R. H. Hruban, R. E. Wilentz, and S. E. Kern, *Am. J. Pathol.* **25** (2000) 579–586.

7. N. R. Hourihan, G. C. O´Sullivan, and J. G. Morgan, *BioTechniques* **30** (2001) 342–346.
8. Z. Yin, R. J. Babaian, P. Troncoso, S. S. Strom, M. R. Spitz, J. J. Caudell, J. D. Stein, and J. Kagan, *Oncogene* **20** (2001) 2273–2280.
9. V. Bazan, I. Zanna, M. Miglia Vacca, M. T. Sanz-Casla, M. L. Maestro, S. Corsale, M. Macaluso, G. Dardanoni, S. Restivo, P. Lopez Quintela, R. Bernaldez, S. Salerno, V. Morello, R. M. Tomasino, N. Gebbia, and A. Russo, *J. Cell. Physiol*. **192** (2002) 286–293.
10. M. Anzola, N. Cuevas, M. Lopez-Martinez, M. Martinez de Pancorbo, and J. J. Burgos, *J. Gastroen. Hepatol.* 19 (2004) 397–405.
11. R. Spaventi, L. Pečur, K. Pavelić, Z. Pavelić, Š. Spaventi, and P. J. Stambrook, *Eur. J. Cancer A.* **30** (1994) 419.
12. J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular cloning: A laboratory manual,* Cold Spring Harbor, New York, 1989.
13. M. Popović Hadžija, S. Kapitanović, S. Radošević, T. Čačev, M. Mirt, D. Kovačević, J. Lukač, M. Hadžija, R. Spaventi, and K. Pavelić, *J. Mol. Med. – JMM* **79** (2001) 128–132.
14. M. Popović Hadžija, R. Hrašćan, M. Herak Bosnar, Ž. Zeljko, M. Hadžija, J. Čadež, K. Pavelić, and S. Kapitanović, *Urol. Res*. **32** (2004*)* 229–235.
15. S. A. Hahn, A. T. M. S. Hoque, C. A. Moskaluk, L. T. da Costa, M. Schutte, E. Rozenblum, A. B. Seymour, C. L. Weinstein, C. J. Yeo, R. H. Hruban, and S. E. Kern, *Cancer Res.* **56** (1996) 490–494.
16. M. Koyama, M. Ito, H. Nagai, M. Emi, and Y. Moriyama, *Mut. Res. Gen.* **406** (1999) 71–77.
17. E. Heinmöller, W. Dietmaier, H. Zirngibl, P. Heinmöller, W. Scaringe, K. W. Jauch, F. Hofstädter, and J. Rüschoff, *Am. J. Pathol.* **157** (2000) 83–92.
18. A. Perren, P. Saremaslani, S. Schmid, C. Bonvin, T. Locher, J. Roth, P. U. Heitz, and P. Komminoth, *Diagn. Mol. Pathol.* **4** (2003) 181–186.
19. M. Litt, X. Hauge, and V. Sharma, *BioTechniques* **15** (1993) 280–284.
20. M. Popović Hadžija, S. Radošević, D. Kovačević, J. Lukač, M. Hadžija, R. Spaventi, K. Pavelić, and S. Kapitanović, *Mut. Res.* **548** (2004*)* 61–73.
21. B. Kozulić, *Food Technol. Biotechnol.* **37** (1999) 45–49.
22. M. Janatova, P. Pohlreich, and B. Matous, *Neoplasma* **50** (2003) 246–250.
23. N. Erill, A. Colomer, M. Calvo, A. Vidal, R. Roman, M. Verdu, C. Cordon-Cardo, and X. Puig, *J. Mol. Diagn.* **7** (2005) 478–485.

SAŽETAK

Novi tip polimera poboljšava karakteristike poliakrilamidnog gela: utvrđivanje gubitka alela

Marina Korolija, Mirko Hadžija, Pajica Pavković, Sanja Kapitanović i Marijana Popović Hadžija

Cilj rada je poboljšati metodu detekcije heterozigota i otkrivanja gubitka heterozigotnosti korištenjem novog polimera. Testirano je 60 uzoraka normalnog i odgovarajućeg tumorskog tkiva debelog crijeva. Umnoženi segmenti razdvojeni su elektroforezom na dva tipa gela: standardnom akrilamid / *N,N*-metilenbisakrilamid gelu te na istom gelu koji sadrži Spreadex polimer NAB (Native Acrylamide-Bis). Na standardnom gelu detektirano je 36 homozigota zdravog tkiva, dok je elektroforeza istih uzoraka na gelu obogaćenom polimerom otkrila među njima 12 heterozigota. Osim toga, 5 tumora odgovarajućih normala (od ukupno 12) izgubilo je jedan alel, što nije detektirano na standardnom gelu, zbog njegove manje moći razdvajanja. Elektroforeza na gelu s novim polimerom predstavlja jednostavnu, financijski pristupačnu, reproducibilnu i neradioaktivnu metodu za utvrđivanje gubitka heterozigotnosti, čije su dobre karakteristike povećana senzitivnost, potrebna manja količina uzorka za analizu i potpuno razdvajanje odsječaka koji se malo razlikuju u veličini. Primjena polimera bi mogla poboljšati i neke druge dijagnostičke metode.